High power-pulsed Nd:YAG laser as a new stimulus to induce BMP-2 expression in MC3T3-E1 osteoblasts

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Abstract: BACKGROUND AND OBJECTIVE: High-power laser has recently become a physical stimulus for bone regeneration. Little is known about how high-power laser irradiation affects osteoblast differentiation. This study investigated osteoblast responses to high-power laser and combined irradiation with BMP-2 treatment. STUDY DESIGN/MATERIALS AND METHODS: MC3T3-E1 pre-osteoblasts were exposed to laser irradiation, 100 ng/ml BMP-2 or both. Cells were irradiated with a Q-switched, pulsed neodymium-doped yttrium aluminum garnet (Nd:YAG) laser, with a 1,064 nm wavelength and 0.75 W output power under 1.5, 3, or 5 J/cm(2) energy densities. Cell proliferation was evaluated using tetrazolium salt, WST-8. To determine the effect of these treatments on in vitro osteogenesis, we examined alkaline phosphatase (ALP) activity, mineral deposition, and expression of genes associated with osteogenesis. Quantitative real time PCR or ELISA was used to examine cytokine expression. In each experiment, either non-irradiated or BMP-2 (100 ng/ml)-treated cells were used as controls. RESULTS: High-power, low-level, Nd:YAG laser irradiation significantly increased ALP activity, when combined with BMP-2 or not. Cell proliferation declined in the irradiation and combined irradiation/BMP-2 groups. Interestingly, Nd:YAG laser stimulation resulted in significant induction of endogenous BMP-2 protein and gene expression. The increased expression of upstream regulators cbfa1 by Nd:YAG laser alone was comparable to exogenous BMP-2 treatment (100 ng/ml). Combined laser/BMP-2 treatment was synergistic in the expression of some genes (IGF-1, cbfa1) and ALP activity, compared to both BMP-2 treatment and laser irradiation alone. In vitro matrix mineralization was significantly accelerated by laser stimulation compared to that of the control, more so than with the combined laser/BMP-2 treatment. CONCLUSIONS: The present in vitro findings demonstrate that high-power, low-level Nd:YAG laser increased osteoblast activity, very efficiently accelerating mineral deposition. Osteoinductive effect of laser is likely mediated by activation of BMP-2-related signaling pathway.

DOI: https://doi.org/10.1002/lsm.20870

High Power-Pulsed Nd:YAG Laser as a New Stimulus to Induce BMP-2 Expression in MC3T3-E1 Osteoblasts

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Key words: bone; BMP-2; high-power laser; Nd:YAG; osteoblast differentiation

ABSTRACT

Background and Objective: High-power laser has recently become a physical stimulus for bone regeneration. Little is known about how high-power laser irradiation affects osteoblast differentiation. This study investigated osteoblast responses to high-power laser and combined irradiation with BMP-2 treatment.

Study Design/Materials and Methods: MC3T3-E1 pre-osteoblasts were exposed to laser irradiation, 100 ng/ml BMP-2 or both. Cells were irradiated with a Q-switched, pulsed neodymium-doped yttrium aluminium garnet (Nd:YAG) laser, with a 1,064 nm wavelength and 0.75 W output power under 1.5, 3 or 5 J/cm^2 energy densities. Cell proliferation was evaluated using tetrazolium salt, WST-8. To determine the effect of these treatments on *in vitro* osteogenesis, we examined alkaline phosphatase (ALP) activity, mineral deposition, and expression of genes associated with osteogenesis. Quantitative real time PCR or ELISA was used to examine cytokine expression. In each experiment, either non-irradiated or BMP-2 (100 ng/ml)-treated cells were used as controls.

Results: High-power, low-level, Nd:YAG laser irradiation significantly increased ALP activity, when combined with BMP-2 or not. Cell proliferation declined in the irradiation and combined irradiation/BMP-2 groups. Interestingly, Nd:YAG laser stimulation resulted in significant induction of endogenous BMP-2 protein and gene expression. The increased expression of upstream regulators cbfa1 by Nd:YAG laser alone was comparable to exogenous BMP-2 treatment (100 ng/ml). Combined laser/BMP-2 treatment was synergistic in the expression of some genes (IGF-1, cbfa1) and ALP activity, compared to both BMP-2 treatment and laser irradiation alone. *In vitro* matrix mineralization was significantly

accelerated by laser stimulation compared to that of the control, more so than with the combined laser/BMP-2 treatment.

Conclusions: The present *in vitro* findings demonstrate that high-power, low-level Nd:YAG laser increased osteoblast activity, very efficiently accelerating mineral deposition. Osteoinductive effect of laser is likely mediated by activation of BMP-2-related signaling pathway.

INTRODUCTION

Non-ablative, low-level laser therapy (LLLT) has been reported to have bio-stimulatory effects for a range of medical applications in skin, nerve, and skeletal muscle tissues [1-3]. Though most laser irradiation applications have focused on soft tissue regeneration [4], recent reports of LLLT stimulating bone metabolism have serious clinical implications in fracture healing and inflammatory bone disease [5,6]. Thus far, laser irradiation is used only as a physical therapy for bone loss, in addition to mechanical loading treatments [7], ultrasound modality [8] and electromagnetic field exposure [9]. Unique among these treatments, laser irradiation can be limited to a desired target region. This targeting may be advantageous and may maximize bone formation efficiency in application to a restricted region such as a dental implant.

While low-level laser irradiation by a low-power semiconductor, such as a diode laser, has shown bio-stimulatory effects, high-power lasers have mainly been used to destroy surfaces, especially those with dental caries [10]. Neodymium-doped yttrium aluminium garnet (Nd:YAG) laser irradiates with a short pulse width and high peak power. This short pulsed, high-intensity laser can produce acoustic waves in the target surface, rapidly heating the tissue [11]. Because wavelength is related to the light penetration through biological tissue [12], 1,064 nm wavelength light from a Q-switched Nd:YAG laser is assumed to reach bone tissue or the marrow cavity through the skin and muscle layers [13]. Recent studies of Nd:YAG lasers in hard tissue applications showed a stimulating effect on bone formation, although there has been relatively little research conducted, as compared to that for low-power lasers, which has been shown to be effective in stimulating bone formation [13,14].

Furthermore, the cellular/molecular mechanism of laser irradiation, both for low-intensity as well as high-intensity lasers, is still in the early stage of study. Intracellular chromophores such as porphyrins and cytochromes absorb light at a low radiation dose, and activated photoreceptors propagate cellular responses [15]. Ninomiya et al. suggested, however, that 1,064 nm light does not have a photochemical influence on bone cells, and that, therefore, it is unknown which receptors sense the signal [14].

Bone-stimulating effects of certain stimuli have been evaluated by assessing alkaline phosphatase (ALP) activity, a key enzyme in early differentiation, or extracellular matrix mineral deposition, an index of late differentiation. Regulating proliferation can be important in bone adaptation to a stimulus, since the balance between these processes determines the size and activity of the osteoblast population at any given time [16]. Another response of interest is the response of the osteoblasts to mechanical stress, in which they produce many osteoblast growth factors; including TGF-β, fibroblast growth factor–2 (FGF-2) and insulin-like growth factors (IGFs) [17]. These factors, then, may be presumed to act as autocrine or paracrine stimulators on bone cells. Shimizu et al. demonstrated that stimulation of bone nodule formation by low-intensity laser irradiation using Ga-Al-As laser (830 nm) was at least partly mediated by IGF-I expression [18]. Therefore, we hypothesized that one major response to high peak power laser irradiation is cytokine release from recipient cells. In this study, we examined osteoblast activity after laser irradiation by determining cell proliferation, ALP activity, mineral deposition, and osteogenesis-related cytokine expression in mouse preosteoblast MC3T3-E1 cells. All activities were investigated under laser irradiation alone and combined with BMP-2 treatment.

MATERIALS AND METHODS

Experimental design of laser irradiation

Laser irradiation was delivered with a high peak power, Q-switched, pulsed Nd:YAG laser apparatus (Fotona D.D., Ljubljana Slovenia). A 0.3 mm diameter optical fiber delivered the laser beam, allowing for uniform irradiation over the cell layer. Laser beam power densities (1.5, 3 and 5 J/cm^2) were determined by varying exposure time (4-12 seconds) over a 2.01 cm^2 area (produced by holding the laser source 3 cm from the cell monolayer). The 0.75 W output power, 15 pps pulse repetition rate and 1,064 nm wavelength remained constant. The laser irradiation experimental conditions are shown in Table 1. Laser irradiation was performed once per day for 7 days in ALP activity assay and mineral deposition or the duration of the study in the other experiments. Each day, control plates were placed on a clean, non-irradiated bench during the laser exposure of the experimental group.

Cell culture

MC3T3-E1 pre-osteoblasts were cultured in growth medium supplemented with minimal essential media modified form (α-MEM; JBI, Korea), 10% heat inactivated FBS (HIFBS), 100 unit/ml penicillin G and 100 μg/ml streptomycin (GIBCO BRL, USA) under cell culture conditions of 37°C in a 5% humidified CO_2 air environment. Osteogenic studies were performed by plating at a cell density of 1.7 x 10^4 cells/cm^2 and culturing in differentiation medium supplemented with α-MEM, ascorbic acid (50 μg/ml), and β-glycerophosphate (10 mM). Non-glycosylated human recombinant BMP-2 was used at the

concentration of 100 ng/ml.

Proliferation test

Cell proliferation was assessed after 2 days of culture by Cell Counting Kit 8 (CCK-8), a simplified assay employing the tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-*2H*-tetrazolium, monosodium salt]. MC3T3-E1 osteoblasts were plated at a cell density of 2,500 cells/well in a 96-well plate. To determine proliferation, 10-μl CCK-8 solutions were directly added to the culture well and incubated for 2.5 hours. The amount of yellow colored product was measured at 450 nm using a microplate reader and is directly proportional to the number of viable cells in a culture media. Each test was repeated in triplicate with four independent samples (n=4).

ALP activity assay

Cells were inoculated at 17,000 cells per cm^2 in 24-well culture plates and cultured in differentiation medium and exposed to laser irradiation, BMP-2 treatment (100 ng/ml) or both when cells reached confluence. Cell lysates were extracted by adding 0.5 ml ALP buffer solution (1.5 M). ALP activity was determined by measuring ρ-nitrophenol production using ρ-nitrophenol phosphate substrate. Cell lysates (20-30 ng) were mixed with ALP buffer solution, gently shaken for 10 min. Then, ALP substrate (17 μM) was added, gently shaken at room temperature for 30 min. After stopping the reaction by adding 0.05 N NaOH, the absorbance was read at 405 nm and compared to a standard curve for ρ-nitrophenol standard solution. Enzyme activity (nmole/protein (mg)/min) was normalized to protein concentration, which was measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA).

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Cells were harvested from day 1 and 2 cultures after successive laser irradiation for two days. Total RNA was extracted by adding 0.5 ml of TRizol reagent. One μg RNA was used for cDNA synthesis with SuperScriptTM Reverse Transcriptase II and oligo12-18 primers. SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used for qPCR assays which were carried out in triplicate on an ABI Prism 7000 sequence detection system (Applied Biosystems, CA). The thermocycling conditions were pre-denaturation at 95°C for 10 min, amplification using 30 two-step cycles of denaturation at 95°C for 15 s, followed by annealing and extension at 60°C for 1 min, with a final dissociation cycle at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted CT). Gene expression values were calculated based on the comparative ΔΔCT method (separate tubes) detailed in manufacturer's instructions. Target genes were normalized to the reference housekeeping gene at glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Fold differences were calculated for each treatment group using normalized CT values for the negative control at the appropriate time point as the calibrator. Mouse-specific oligonucleotide primers for RT-PCR were designed with product sizes less than 200 bp using Real-Time PCR system Sequence Detection Software v 1.3 (Applied Biosystems, CA, USA). The mouse primer sequences were as follows: IGF-1(GeneBank# AF440694)forward- TGG ATG CTC TTC AGT TCG TG, reverse-CTT CAG TGG GGC ACA GTA CA; VEGF-A (GeneBank#M95200) ; forward-AGA GCA ACA TCA CCA TGC AG, reverse-AAT GCT

TTC TCC GCT CTG AA; BMP-2 (GeneBank# NM_007553) forward-TGG AAG TGG CCC ATT TAG AG, reverse-TGA CGC TTT TCT CGT TTG TG; cbfa1 (Genebank#NM009820) forward- CCA GGA AGA CTG CAA GAA GG, reverse- AGG CTG TTT GAC GCC ATA GT; osterix (GeneBank#X13409) forward- GGA GGC ACA AAG AAG CCA TA, reverse- GGG AAG GGT GGG TAG TCA TT; dlx5 (GeneBank#U67840) forward- TCT CAG GAA TCG CCA ACT TT, reverse- GAG CGC TTT GCC ATA AGA AG; GAPDH (GeneBank# M32599) forward- ACT CCA CTC ACG GCA AAT TC, reverse- TCT CCA TGG TGG TGA AGA CA.

Enzyme-linked immunosorbent assay (ELISA) of BMP-2

BMP-2 levels in culture supernatants were determined using ELISA kits (Quantikine®, R&D Systems, USA) according to the manufacturer's instructions. Cell culture supernatants at days 1 and 2 after laser irradiation in growth medium were added to 96-well ELISA plates after centrifugation. BMP-2 standards (0-20,000 pg/ml) were run in each series. Following incubation, aspiration, and washing, mouse/rat/human BMP-2 conjugates (200 μl/well) were added per the manufacturer's instructions. The optical density of each well was determined within 30 min using a microplate reader set to a 450-nm wavelength correction for optical imperfections in the plate. The ELISA was repeated in triplicate with three or four independent samples (n=3-4).

Determination of matrix mineralization; Alizarin red S & von Kossa staining

Calcium deposition was determined using Alizarin red S staining. MC3T3-E1 osteoblasts were irradiated once a day for seven days and further cultured in osteogenic

medium. After 12 days, cells were fixed in 70% ice-cold ethanol for 1 h, rinsed with double-distilled H_2O, and stained with 40 mM Alizarin red S (pH 4.2) for 10 min with gentle agitation. After five washes with double-distilled H_2O, the cells were rinsed for 15 min with 1x PBS with gentle agitation to remove any cells experiencing non-specific binding.

To perform von Kossa staining cells were cultured for 21 days under the same conditions as for Alizarin red S staining. Then, the cells were washed with PBS, fixed in 10% formaldehyde solution for 10 min, and washed in deionized water. The fixed cells were incubated with 5% silver nitrate solution for 30 min in the dark and rinsed in deionized water. The cells were treated with sodium-carbonate formaldehyde solution for 2 min, and then incubated with staining solution (5% sodium thiosulphate) for 20 min after rinsing in deionized water. Finally, the cells were washed in deionized water and left to air dry.

Statistical analysis

All data are presented as mean ± standard error of mean (SEM). Each experiment was performed at least three times ($n \geq 3$ per group). Final mean value of each group was calculated from mean values of each sample, followed by averaging all triplicates of each sample. Statistical analysis was performed using SAS 9.1.3 software (SAS Institute Inc. Cary. NC. USA). One-way analysis of variance (ANOVA) and the post hoc multiple comparison Tukey's test as well as nonparametric Kruskal-Wallis test were applied to determine significance among experimental groups. Results were considered statistically significant at $p < 0.05$.

RESULTS

1. Cell proliferation

Irradiated and non-BMP-2-treated cells were seeded onto separate plates to avoid indirect irradiation from laser beam exposure to neighboring wells. In addition, irradiated cells were seeded in alternate wells of the same culture plate because the irradiation area exceeds that of a 96-well plate well. On day 3, cell proliferation was significantly suppressed by 13.6 %, 11.2 % and 7.5 % in the irradiated groups of 1.5, 3 and 5 J/cm^2 energy intensities, respectively, compared to that of the control (non-treated) cells (Fig. 1). BMP-2 treatment (100 ng/ml) resulted in a minor trend toward increased proliferation, but without significance. Combined laser/BMP-2 treatment did not restore the suppressed proliferation to the untreated or BMP-2-treated levels.

2. ALP activity and mineral deposition

The effect of laser irradiation on osteoblast differentiation was evaluated by assessing ALP activity, a measure of early differentiation, and mineral deposition using both Alizarin red S and von Kossa staining, a measure of late phase differentiation. ALP activities were assessed at 3, 7 and 14 days in culture in differentiation medium supplemented with AA and β-GP or additional BMP-2 treatment (100 ng/ml) (Fig.2). Cells were irradiated once a day for seven days at 1.5, 3 and 5 J/cm^2 energy intensities either with or without BMP-2. Laser irradiation promoted a significant increase in ALP activity by 65%, 54% and 24% at day 3 and 110%, 82% and 80% at day 7 at 1.5, 3 and 5 J/cm^2 power intensities, respectively, compared to that of the untreated controls. Cells irradiated at 1.5 J/cm^2 showed a 45%

increase in ALP activity at day 14 with a significance. BMP-2 treatment led to a time-dependent, significant increase in ALP activity by 50% (p<0.01), 50% (p=0.05) and 162% (p=0.013) at day 3, 7 and 14, respectively. Combined laser irradiation/BMP-2 treatment increased ALP activity in all groups at 1.5, 3 and 5 J/cm^2 energy intensities more than the BMP-2 treatment by 76%, 87% and 90% at day 3, and 104%, 123% and 124% at day 7, or more than laser irradiation by 60%, 82% and 126% at day 3 and 48%, 87% and 89% at day 7, respectively, with a significance (p<0.01). Combined stimulation on day 14 decreased ALP activity less than BMP-2 treatment alone, but still higher than laser irradiation alone by increase of 22%, 100% and 96% at 1.5, 3 and 5 J/cm^2 energy intensities, respectively, with significance (p<0.01).

Mineral deposition was evaluated using Alizarin red S staining at day 12 and von Kossa staining at day 21 under the same culture conditions as the ALP activity assay (Fig. 3). Alizarin red S staining showed highly intensified calcium deposition in the irradiation alone group, more than in the untreated, BMP-2 alone or combined irradiation/BMP-2 treatments. Delayed observation with von Kossa staining at day 21 followed a similar trend as in the Alizarin red S staining. Interestingly, *in vitro* mineralization was more efficient in each irradiated group than in the combined irradiation/BMP-2 treatment, in both the Alizarin red S and von Kossa stained cells. Statistical analysis determined by measuring the mineralized area after von Kossa staining showed two-fold increases in mineralization in the groups irradiated at 1.5 and 5 J/cm^2 energy intensities versus the combination of irradiation/BMP-2 treatment, and 1.5 fold increase at 3 J/cm^2 (Fig. 3B)

3. Gene expression of osteogenesis-related cytokines

Bone tissue requires blood vessel formation within the bone microenvironment. VEGF is an important cytokine for angiogenesis, and thus acts as a potent stimulator of bone formation, along with BMP-2 and IGF-1. The effects of irradiation on proliferation and ALP activity were similarly independent of dosage in the range of 1.5 to 5 J/cm^2 energy density. Energy density at 3 J/cm^2 was chosen to assess cytokine release from cells with or without BMP-2 treatment because IGF-1 induction was reported at the similar energy density using GA-Al-As laser [18]. IGF-1, BMP-2 and VEGF expressions were evaluated using qRT-PCR at days 1 and 2 (Fig. 4A, B and C). Laser irradiation significantly increased BMP-2 and IGF-1 expression by 212% (p=0.0017) and 205% (p=0.0038), respectively, compared to that of the untreated cells at day 2. BMP-2 treatment alone resulted in significantly up-regulated IGF-I expressions by 71% (p=0.0038) at day 2 while it did not affect endogenous BMP-2 or VEGF expression at day 1 and even reduced BMP-2 expression at day 2. Combined irradiation/BMP-2 treatment led to day 2 synergy in IGF-1 and VEGF expression, compared to either BMP-2 or laser treatment alone. This combination resulted in an increase of 180 % (p=0.0415) and 130 % (p=0.07) at VEGF expression, and of 81.5 % (p<0.001) and 38.5 % (p=0.004) at IGF-1 expression in comparision with BMP-2 treatment and laser irradiation, respectively. Decreased endogeneous BMP-2 expression by BMP-2 treatment at day 2 could be recovered by combined treatment of BMP-2 with laser irradiation, resulting in a significant increase by 120 % (p=0.0039) over that in BMP-2-treated group. Irradiation dose-independently increased extracellular BMP-2 secretion in all irradiated groups at 1.5, 3 and 5 J/cm^2 by around 17% with a significance (p<0.001), compared to that of the untreated control.

4. Gene expression of BMP-2 responsive transcriptional factors

To determine whether laser irradiation affects expression of upstream regulators of BMP-2 responsive transcriptional factors, we examined cbfa1, osterix and dlx5 expressions using qRT-PCR (Fig. 5). These genes were significantly up-regulated by either BMP-2 treatment or laser irradiation. Laser irradiation significantly increased cbfa1 and osterix expressions by 280% (p=0.0041) and 11% (p=0.039) only at day 1, while it did not affect dlx5 expression at any tested time. Among these factors, irradiation affected the cbfa1 more than it did the osterix. BMP-2 treatment led to a significant increase in cbfa1, dlx5 and osterix gene expressions at day 1 or 2. In BMP-2 treated group, Cbfa1 expression was higher than that in combination with laser irradiation, and osterix and dlx5 expression were equivalent to those in combined treatment at day 1. The effect of combined irradiation/BMP-2 treatment at day 1 did not appear in all tested gene expressions, compared with that of BMP-2 treatment, but only osterix expression showed a significant increase of 170% (p=0.029) over laser irradiated group. Combined irradiation/BMP-2 treatment at day 2 resulted in a differential synergistic increase, depending on the gene. Combined treatment significantly increased cbfa1 expression by 80.1 % (p<0.001) and 38.5 % (p=0.0032), compared to both BMP-treatment and laser irradiation, respectively, while dlx5 and osterix gene expression showed a significant increase only over laser irradiated group by 140% (p=0.0017) and 290% (p=0.0035), respectively

DISCUSSION

Recent study of laser irradiation has extended to bone and cartilage regeneration due to successful outcomes in soft tissue therapy. Laser apparatuses have technologically improved by varying parameters for clinical applications. The Q-switched, pulsed Nd:YAG laser, also known as a surgical laser, has the advantage that it does not heat the treated tissue. It is also requires only a brief exposure time to reach the desired intensity. Furthermore, Nd:YAG laser light with a wavelength of 1064 nm may influence bone formation because light with a wavelength of 600 nm to 1100 nm projected through the skin and muscle layer is supposed to reach the bone tissue or marrow cavity in high energy density [12-14]. Thus, we focused on characterizing the effect of high power laser irradiation on cell response in relation to bone metabolism, despite its general acceptance as a destructive surgical laser. The present study revealed that high-power, low-level Nd:YAG laser was able to favor osteoblast differentiation through increase of ALP activity and mineral deposition. This stimulus activated BMP-2-related signaling pathway.

In vitro laser irradiation can be readily delivered to a cell layer by a movable optical fiber.Cells were exposed to a desired energy by maintaining a certain distance between the laser beam and the recipient cells, and varying the exposure time for each energy density. Despite accumulated information about the effects of total energy dose, laser spectrum, energy density and irradiation phase, it is still unclear which of these parameters have the greatest effect on therapeutic efficiency, even in low-level lasers which have shown diverse bio-stimulatory effects [4]. Although the energy density and exposure time in He-Ne laser irradiation have been reported to be more important than the total energy dose in the

stimulation of fibroblast proliferation and collagen production [19], that result is still controversial. The present study did not compare all of these parameters; however, the bio-stimulatory effect of high power laser (0.75 W) on differentiated osteoblasts was investigated at energy densities ranging from 1.5 to 5 J/cm^2. These energy densities belong to the range of low-level laser irradiation. High power output (0.75 W) allowed a relatively short exposure to a 24-well plate (4 s for 1.5 J/cm^2, 8 s for 3 J/cm^2, and 12 s for 5 J/cm^2).

Low-level laser therapy (LLLT) has been shown to stimulate cellular proliferation, differentiation, collagen synthesis and growth factor release from cells, depending on the cell type [18,20-22]. The proliferative effect of laser stimulation is important in bone formation for the enhancement of bone density. In the present study, high-power Nd:YAG laser irradiation suppressed cell proliferation at common low-level doses (1.5-5 J/cm^2). A similar dose range (1-4 J/cm^2) for Ga-Al-As diode laser irradiation, using either visible red (660 nm) or infrared lasers (780 nm), reportedly showed a positive biostimulatory effect on fibroblast and osteoblast proliferations [20-23]. Despite several differences between these studies, including wavelength, irradiation area, irradiation mode etc., the main difference is of the powers, 750 mW (high) vs. 20/40 mW (low). Another report using a Nd:YAG laser showed similar results, power output over 0.6 W has a dose-dependent, suppressive effect on cell proliferation [24]. Therefore, high power output may be unfavorable for cell proliferation or induce initial cell cycle arrest, as reported in experiments using Ga-Al-As diode laser [25]. The main LLLT controversy is in the difference between *in vitro* and *in vivo* studies. *In vivo* LLLT studies using Ga-Al-As diode laser on bone tissue regeneration failed to find an effect in the canine palate animal model, while laser irradiation with common parameters stimulated both cell proliferation and differentiation *in vitro* [26,27]. Therefore, the dose at the target

region *in vivo* may be lower than that of the instant beam due to absorption or scattering by erythrocytes or microvessels [28].

In contrast to the suppressed cell proliferation, Nd:YAG laser facilitated osteoblast differentiation. It showed enhanced early and late stage osteogenic activities, as determined by ALP activity and *in vitro* matrix mineralization, respectively. Mineral deposition induced by laser irradiation was higher than that of the combined laser/BMP-2 treatment, while the combined treatment effect is greater than each of the individual effects in ALP activity. BMP-2 treatment stimulated very little *in vitro* mineralization, and combining laser and BMP-2 treatments reduced the laser-mediated increase. Luppen et al. reported similar findings in which BMP-2 administration dose-dependently inhibited mineralization in MC3T3-E1 cells [29]. This intriguing result was explained by either BMP-2 feedback inhibition or relatively weak BMP-2 activity in the differentiated cells. Our previous report using human mesenchymal stromal cells (hMSCs) showed a similar trend; BMP-2 treatment did not accelerate *in vitro* mineralization while it induced other activities, such as ALP or gene expression of osteogenesis-related markers [30]. A common finding in the present and previous studies using hMSCs was that BMP-2 is highly efficient in early osteoblast differentiation while it has a weak activity in late differentiation. Although accumulated *in vivo* and *in vitro* information leaves no doubt about the osteogenic efficacy of BMP-2, the *in vitro* matrix mineralization findings remain controversial and suggest additional requirements for matrix mineralization. Laser irradiation was very effective from the early phase to the late phase of osteoblast differentiation.

Of the bio-stimulatory effects of bone formation that are activated by physical stimulations or stress, cytokine release is most important in the regulation of local bone cell function in an autocrine or paracrine fashion [31]. Studies characterizing cellular proliferation and differentiation stimulated by laser irradiation address cytokine or growth factor release as regulators. Low intensity Ga-Al-As laser irradiation (3.82 J/cm^2) was reported to significantly increase IGF-1 protein and gene expressions, which partly contribute to accelerated matrix mineralization [18]. Another report showed that a high energy Ga-Al-As laser irradiation increased BMP-2 and -4 expressions in a range of weak doses in human dental pulp cells [22]. Our study focused on changes in cytokine gene expression, which plays a critical role in bone metabolism. High-power, low-level Nd:YAG laser irradiation up-regulated the gene expression of cytokines, including IGF-1 and BMP-2, while it did not affect VEGF expression. Laser treatment alone significantly increased BMP-2 gene expression and extracellular release at low-level intensities. These results indicate that laser-induced BMP-2 production may mediate the resulting increase in ALP activity and *in vitro* matrix mineralization.

Studies of upstream regulation of laser-mediated BMP-2 induction found that laser irradiation alone significantly up-regulated the BMP-2-specific transcriptional regulators, cbfa1, osterix and dlx5. Among these factors, cbfa1expression has a longer sensitivity to laser stimulation. The expression of osterix and dlx5 showed an additive effect in combined laser/BMP-2 treatment, whereas it did not respond to laser stimulation. A weak synergy in cbfa1 expression of BMP-related transcription factors indicated that laser and BMP-2 treatments followed similar, perhaps overlapping, cell stimulation pathways. The study of the main regulation pathway in laser irradiation has focused on the function of intracellular

receptors, chromophores which sense the photogenic energy of laser irradiation and transfer it to chemical energy within the cells [32]. The resulting physiological changes mediate cell responses in a range of cells [33]. Responsible chromophores vary depending on wavelength. The over 1,064 nm wavelengths produced by a Nd:YAG laser are sensed by the chromophore, hemoglobin, which is unlikely to influence bone cells [14]. Therefore, acoustic or shock waves have been proposed as the inducing stimulation resulting from high power Nd:YAG laser exposure, rather than the normal photochemical stimulation [12]. Some reports demonstrate that shock waves induce BMP-2 or BMP-2-inducible kinase expression [34,35]. The present finding that cbfa1 expression is sensitive to laser irradiation provides a supporting evidence for the potential role of laser irradiation as a mechanical signal, based on the report that cbfa1 is a target of mechanical signals in osteoblastic cells [36].

In conclusion, irradiation using high-power Nd:YAG laser promoted osteogenic activities, including ALP activity, *in vitro* matrix mineralization and cytokine expression, some of which are comparable to exogeneous BMP-2 treatment. Irradiation, however, suppressed cell proliferation even at power densities under 5 J/cm^2 where other lasers usually increase proliferation. Increased BMP-2 and upstream regulator, cbfa1expression may play an important role in high-power Nd:YAG laser stimulating osteoblast differentiation. Combined high-power Nd:YAG laser/BMP-2 stimulation showed a synergistic effect on the some gene expressions over laser irradiation, BMP-2 treatment alone or both. Further study with varied BMP-2 doses or laser stimulation input intervals will be necessary to determine if synergy in osteogenesis stimulation can be achieved. Our findings suggest that high power Nd:YAG laser enhanced bone formation *in vitro*, highlighting the application as a tool for target-oriented osteogenic therapy.

ACKNOWLWDGEMENTS

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (A084359). The author group thanks Shinhan MNT (Seoul, Korea) for providing the laser apparatus.

Table 1. Experimental conditions of laser irradiation

Groups	Output power (W)	Pulse repetition rate (pps)	Total irradiation time (s)	Total energy density (J/cm^2)
Group 1	0.75	15	4	1.5
Group 2	0.75	15	8	3
Group 3	0.75	15	12	5

Energy densities (1.5, 3 and 5 J/cm^2) were determined by varying exposure time (4-12 seconds) over a 2.01 cm^2 area which was produced by holding the laser source 3 cm from the cell monolayer.

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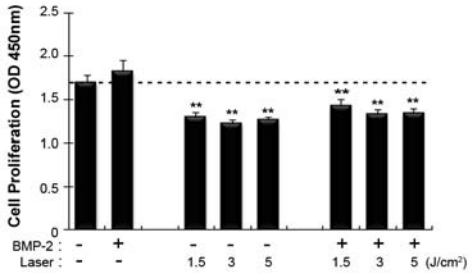

Figure 1. Nd:YAG effect on MC3T3-E1 osteoblast proliferation. Cell proliferation (n=5) was measured using cell proliferation reagent WST-8 at day 3 after laser irradiation with or without BMP-2 (100 ng/ml). Cells were exposed to laser irradiation (once/day) at 1.5, 3 and 5 J/cm^2 energy densities. Significantly different from control, p^{**}<0.01.

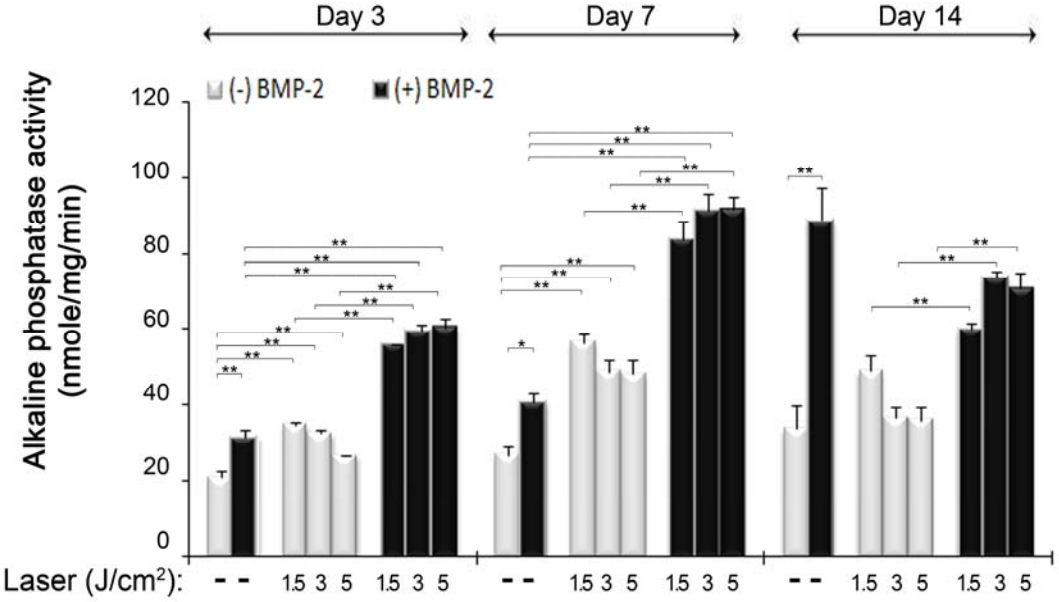

Figure 2. Alkaline phosphatase (ALP) activity after laser irradiation, BMP-2 treatment or combined irradiation/BMP-2 treatment. Cells were exposed to laser irradiation (once/day) at 1.5, 3 and 5 J/cm^2 energy densities for 7 days with or without continuous BMP-2 treatment (100 ng/ml). ALP activity (nmole/min/mg protein) was measured at days 7, 14 and 21 after culturing in osteogenic differentiation medium. Significantly different from the control, or from combined irradiation/BMP-2 treatment, p^{*}<0.05, p^{**}<0.01.

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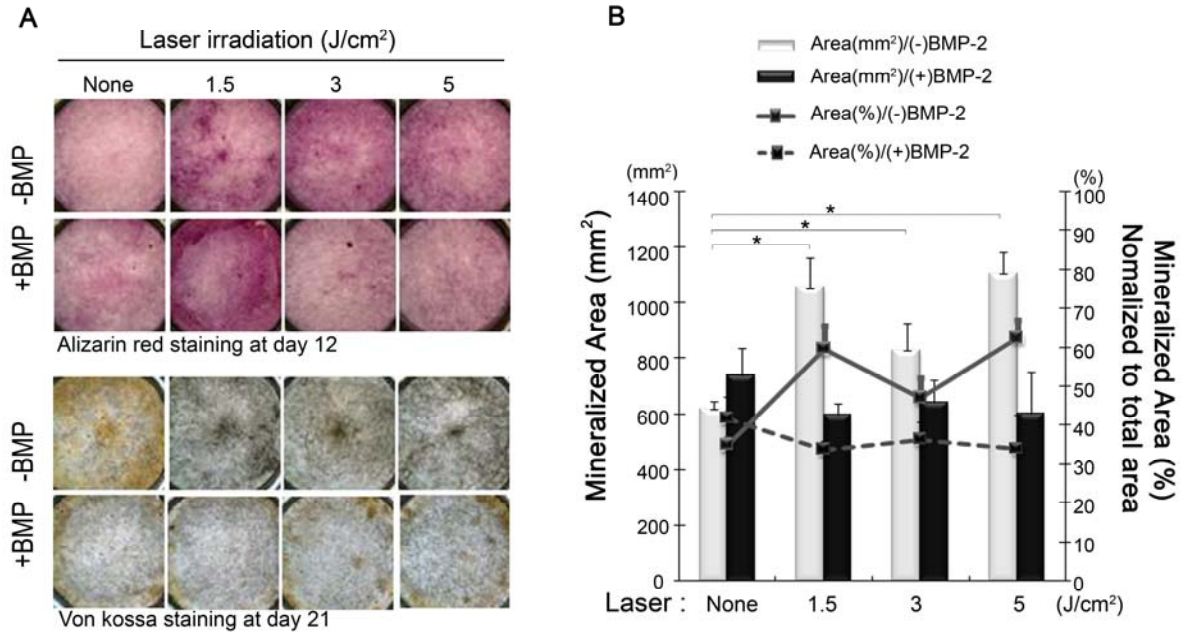

Figure 3. Nd:Yag effect on *in vitro* mineralization. (A) Osteoblasts were cultured in the same conditions as in the ALP activity assay. Mineral deposition was visualized by Alizarin red S staining after 12 days in culture or von Kossa staining after 21 days in culture. Images shown are representative of three independent experiments. (B) Matrix mineralization was quantified by image analysis of four representative 1.25x fields for each treatment using Spot software after von Kossa staining. Mineralized areas are represented as area (mm^2) or percentage (%) normalized to total area. Statistical significance between control and experimental groups or between B/L and L group, $p^{*}<0.05$, and $p^{**}<0.01$.

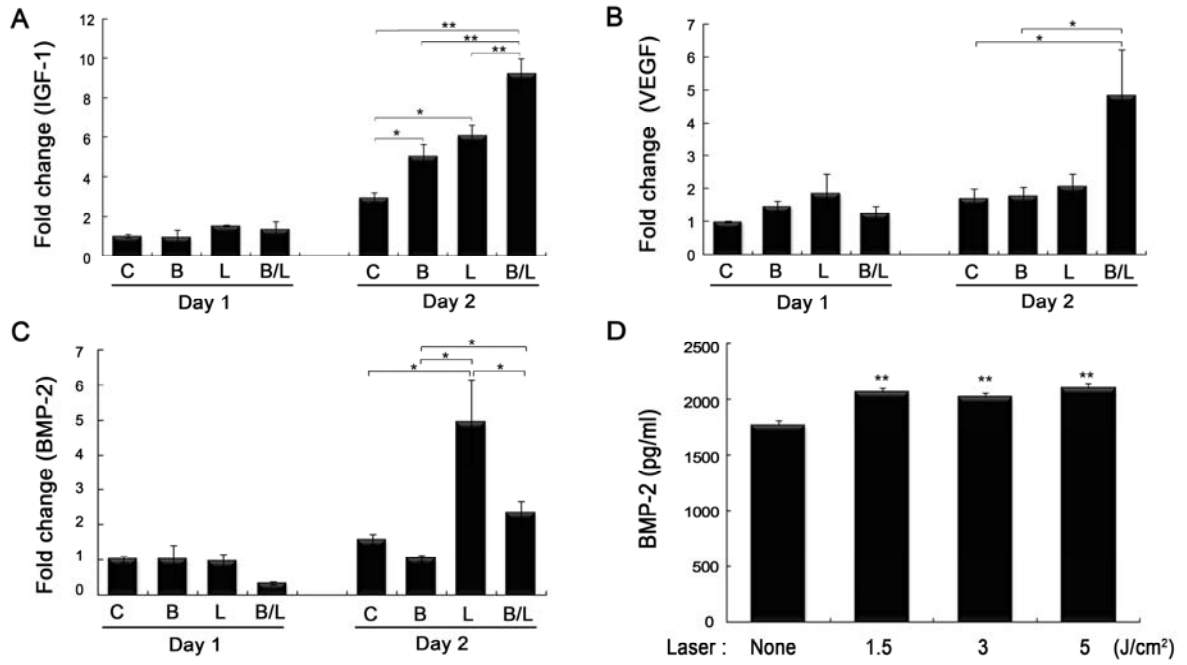

Figure 4. Nd:YAG effect on gene expression of osteogenesis-associated cytokines. qRT-PCR analysis was performed for *IGF-1* (A), *VEGF* (B) and *BMP-2* (C of subfigure) after days 1 and 2 in culture of control (C), or with BMP-2 treatment (B), laser irradiation (L), or combined BMP-2/irradiation treatment (B/L). *Statistical significance between control and experimental groups or between B/L and B or L group, $p^{*}<0.05$, and $p^{**}<0.01$. (D) Extracellular BMP-2 release was measured by ELISA at day 2 after laser irradiation (once/day) at 1.5, 3 and 5 J/cm^2 energy densities. Statistical significance between control and experimental groups or between B/L and B or L group, $p^{*}<0.05$, and $p^{**}<0.01$.

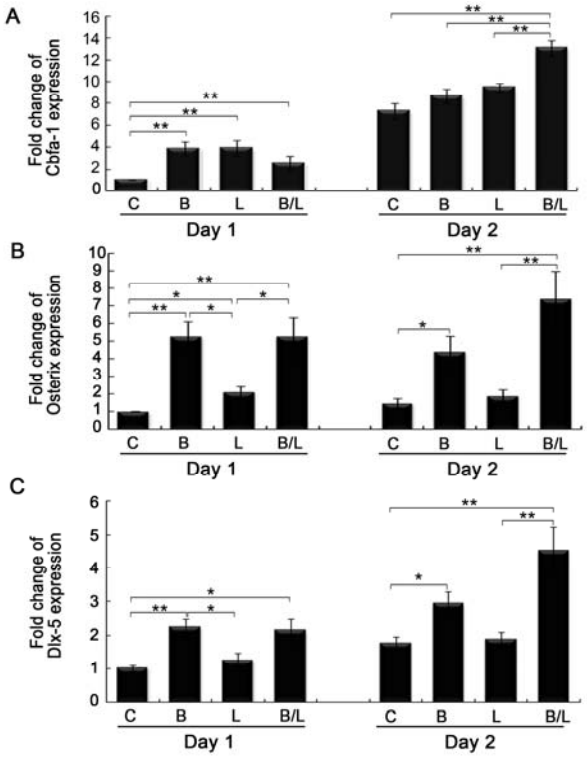

Figure 5. Gene expression of osteoblast-specific transcription factors using qRT-PCR. The *cbfa1*(A), *osterix* (B), and *dlx5* (C) mRNA expression levels were examined in the same conditions as in Fig.4 (A, B, C). Statistical significance between control and experimental groups or between B/L and B or L group, $p^{*}<0.05$, and $p^{**}<0.01$.